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Linoleic acid improves the robustness of cells in agitated cultures

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Abstract

The murine hybridoma (CC9C10) was subjected to high shear rates in a spinner flask to determine the effect of various culture additives on cell survival. At 500 rpm, the half-life of the viable cell concentration in a low protein serum-free medium was 50 min. Both bovine serum albumin and Pluronic F-68 had a significant effect in protecting cells under these conditions. The effects of the two supplements were additive, so that in the presence of both supplements there was minimal cell damage at 500 rpm. The survival rate of cells grown in media supplemented with linoleic acid improved significantly under high stirring rates. Cells grown for one passage in 50 μM linoleic acid and stirred at 500 rpm had a significantly higher survival rate than control cells. For cells grown over 5 passages in 25 μM linoleic acid, the survival rate at 470 rpm was $\times 3$ greater than that determined for control cells. This difference gradually decreased at higher stirring rates up to 610 rpm when the half-life of the viable cell population was reduced to $\sim$10 min. Supplementation of cultures with linoleic acid has previously been shown to result in incorporation into all three cellular lipid fractions – polar, non-polar and free fatty acid (Butler et al., 1997). Our explanation for the increased survivability of the cells at high agitation rates in the presence of linoleic acid is that the structural lipid components of the cell including the outer membrane attained a higher unsaturated/saturated ratio which was more robust than that of control cells.

Introduction

Mammalian cell lines used for large-scale production processes are often adapted to serum-free media (Butler, 1986). This allows batch-to-batch culture consistency, reduces the risk of contamination and eases final protein purification (Cleveland et al., 1983; Kawamoto et al., 1983; Tharakan et al., 1986; Kovar and Franek, 1986; Murakami, 1989). Fatty acids are often included in serum-free media to replace the growth-promoting properties of the lipid component of serum. However, the effect of selected fatty acids on cell growth is variable. Fatty acids have been observed to stimulate (Rose and Connolly, 1990; Grammatikos et al., 1994; Jager et al., 1988), inhibit (Calder et al., 1991) or have no effect on cell growth (Cornwell and Morisaki, 1984; Bailey and Dunbar, 1973; Spector et al., 1981). The concentration dependence of fatty acid stimulation or inhibition has been shown previously for mitogen-stimulated lymphocytes (Calder et al., 1991; Kelly and Parker, 1979; Cuthbert and Lipsky, 1989) and hybridomas (Kovar and Franek, 1986). A low concentration of unsaturated fatty acids (<75 μM) may promote growth whereas a higher concentration (>100 μM) can be inhibitory (Cornwell and Morisaki, 1984; Karsten et al., 1994). Such a sharp concentration optimum may account for apparently contradictory reports of the growth effects of fatty acids in the literature.

Saturated fatty acids or unsaturated fatty acids such as oleic acid (18:1, n-9) can be synthesised by normal mammalian cells that possess elongation and desaturation enzymes (Rosenthal, 1987). However, the polyunsaturated fatty acids of the n-3 and n-6 group such as linoleic acid (18:2, n-6) or linolenic acid (18:3, n-3) are essential nutrients for animals because they are precursors for the synthesis of eicosanoid hormones such as prostaglandins (Needleman et al., 1986). Linoleic acid has been shown to enhance the proliferation of mouse mammary epithelial cells by

Table 1. Metabolic state of cells grown with fatty acids

State 1 →	State 2 ↔	State 3
passage 0	passage 1	passage >5
– low cell yield	– high cell yield	– high cell yield
– low Mab yield	– high Mab yield	– low Mab yield
– lipid-starved	– lipid-balanced	– lipid-loaded

metabolism to arachidonic acid which is a precursor of prostaglandin E_2 (Bandyopadhyay et al., 1987). However, the mechanism of growth-promotion of the unsaturated fatty acids in culture may be related to their importance in the synthesis of cellular membranes (Rockwell et al., 1980; Rintoul et al., 1978) which may have a significant effect on membrane fluidity (Calder et al., 1994).

Unsaturated fatty acids have been shown to be essential for the repeated passage of hybridomas in serum-free cultures. However, this requirement may only become apparent in culture after several passages in serum-free medium following adaptation from serum-based medium (Jager et al., 1988). In previous reports from our lab (Butler and Huzel, 1995; Butler et al., 1997) we showed that linoleic or oleic acid enhances significantly the cell yield and monoclonal antibody productivity of a murine hybridoma. Of 12 fatty acids tested, only oleic or linoleic acid increased the cell yield substantially and significantly. The cell yields were enhanced by as much as $\times 3$ in 25 μM linoleic acid or 50 μM oleic acid compared to fatty acid-free control cultures. The effect of growth enhancement was reversible. When cells that had been passaged continuously in the presence of fatty acids were re-introduced into unsupplemented medium, the growth advantage over control cultures was lost within 3 to 4 passages. The antibody productivity of these cells increased initially with culture supplementation with unsaturated fatty acids. However, there appeared to be a maximal level of fatty acid loading beyond which the antibody production decreased. Continued culture passage (>5) with the unsaturated fatty acids led to a lipid-loaded state in which cells maintained a high capacity for growth but a decreased capacity for antibody production.

These effects can be summarised by identifying 3 distinct metabolic states (Table 1). State 1 was achieved by adaptation of cells from serum-supplemented media for at least 10 passages in serum-free fatty acid-free media. State 2 arises following a brief exposure (1 passage) to linoleic (or oleic) acid (25–50 μM). State 3 arises from prolonged exposure to the fatty acids and can be partially reversed to state 2 following the removal of fatty acids from the growth medium. The fatty acid composition of each of these states is significantly different. In state 1, 82% of cellular fatty acids could be accounted for by palmitic, oleic and stearic acids. The linoleic acid concentration was low (<5%). In state 2 the linoleic content increased to 70% and in state 3 to 90%, whilst the content of the other fatty acids decreased progressively over this period to 5%. Further evidence of metabolic differences between cells in these 3 states comes from the analysis of energy metabolism. As cells progress from state 1 to state 3 there is a change in the pattern of energy metabolism in which the oxidative metabolism of glucose increases and the oxidative metabolism of glutamine decreases. Cells in state 3 grown in the absence of fatty acid revert to state 2 and demonstrate a partial reversal of this oxidative pattern. In all states the rate of fatty acid oxidation was low in relation to the total utilisation of fatty acid and in relation to glucose and glutamine oxidation. This altered metabolic pattern may be attributed to a marked reduction in the transport of glutamine into the cells grown with fatty acids.

This work has shown that the availability of specific fatty acids is one of the key factors in the control of hybridoma metabolism and will affect long-term antibody production in serum-free cultures. It would appear that the cells have a requirement for linoleic and/or oleic acid but are unable to regulate the rate of uptake to maintain maximal cell and Mab yields (state 2 in Table 1).

The possibility that the increased linoleic acid content of structural lipid components of cells could alter the properties of cell membranes led us to investigate the survivability of the cells in agitated cultures. The robustness of the cells under conditions of agitation is important for production processes in large-scale cultures.

Materials and methods

Cells

The murine B-lymphocyte hybridoma (CC9C10), which secretes a monoclonal antibody (IgG_1) against bovine insulin and the parental myeloma (Sp2/0) were

obtained from the American Type Culture Collection (ATCC No HB123 and CRL 1581).

Cultures

The cells were adapted over several passages to a serum-free medium which was previously described (Butler and Huzel, 1995). The cells were grown for at least 10 passages in this medium prior to the described experiments. The serum-free formulation contained either insulin or a recombinant analogue of insulin-like growth factor (Long R^3 IGF). Each was equally effective in promoting cell growth. Growth yields in the presence of either insulin or recombinant IGF were enhanced by 20%. Cultures were established in T-flasks or in 24-well plates and grown under a humidified atmosphere at 10% CO_2. The basal medium contained a low level of methyl linoleate (0.15 μM). However, this is a minimal concentration compared to the linoleic acid supplements used in the experimental cultures.

Fatty acid supplement

The cultures were supplemented with fatty acid-free bovine serum albumin (0.1 mg ml^{-1}) which was complexed with a specific concentration of a fatty acid. A BSA-fatty acid concentrate ($\times$ 100) was formed by mixing 1 ml of BSA (10 mg ml^{-1}) with varying volumes (1–10 μl) of fatty acid (1 M) in ethanol, according to the requirement of each experiment. The concentrate was mixed for 30 min at room temperature prior to addition to the cultures.

Cell counting

Viable cell concentrations were determined by counting a cell suspension diluted 1:1 v/v with 0.2% trypan blue using a Neubauer haemocytometer.

Culture agitation

The agitation experiments were performed with cell suspensions held in 250 ml Bellco spinner flasks containing a flat blade impeller with a diameter of 5 cm. Agitation was maintained by a magnetic stirring base held in an incubator. The speeds of rotation were calibrated by a stroboscope (Model SF-9211; Pasco Scientific).

Curve fitting

Non-linear regression analysis was performed to curve fit the data using SigmaPlot 4.01.

Results

The effect of BSA and Pluronic on cell survival in agitated cultures

In order to develop a simple assay to test the effects of shear stress on cell viability in culture, spinner flasks were agitated at rates well above those normally used for growth. In these spinner flasks a vortex developed in the stirred liquid at agitation rates above 300 rpm. Figure 1 shows the effect of a stirring rate of 500 rpm on the viable cell concentration over a 4.5 h period. The viability of cells in suspension in the basal medium which had a low protein content ($<$20 μg/ml) and in the absence any protectant, decreased rapidly with a half-life of 0.81 h as determined from the decay curve. The addition of BSA (100 μg/ml) to the cultures improved the survival of the cells (half-life = 1.7 h) as did Pluronic (0.1%). The effect of Pluronic increased the half-life to a value of 5.6 h as determined from extrapolation of the decay curve. The addition of both BSA and Pluronic resulted in maximal cell survival with a decrease in viable cell concentration to only 90% of the original value over the period of the experiment.

It is to be noted that the cells were maintained in parallel cultures with or without BSA for several passages prior to the agitation experiment. Pluronic was added to the appropriate cultures 4 days prior to the experiment.

The effect of linoleic acid on cell survival in agitated cultures

The effect of linoleic acid on the survival of cells was determined using the same agitation conditions as previously described. Linoleic acid was complexed to de-lipidated BSA prior to supplementation to the cultures. In this experiment the cells were grown for one passage in the linoleic acid-supplemented medium prior to the test. The results (Figure 2) show a significantly increased survival for cells grown in linoleic acid compared to a control culture containing fatty acid-free BSA. The half-lives of viable cells as determined by a single exponential decay curve were 2.38 and 3.63 h, respectively, for cultures containing 25 μM and 50 μM linoleic acid. This was compared with the control culture half-life of 1.97 h.

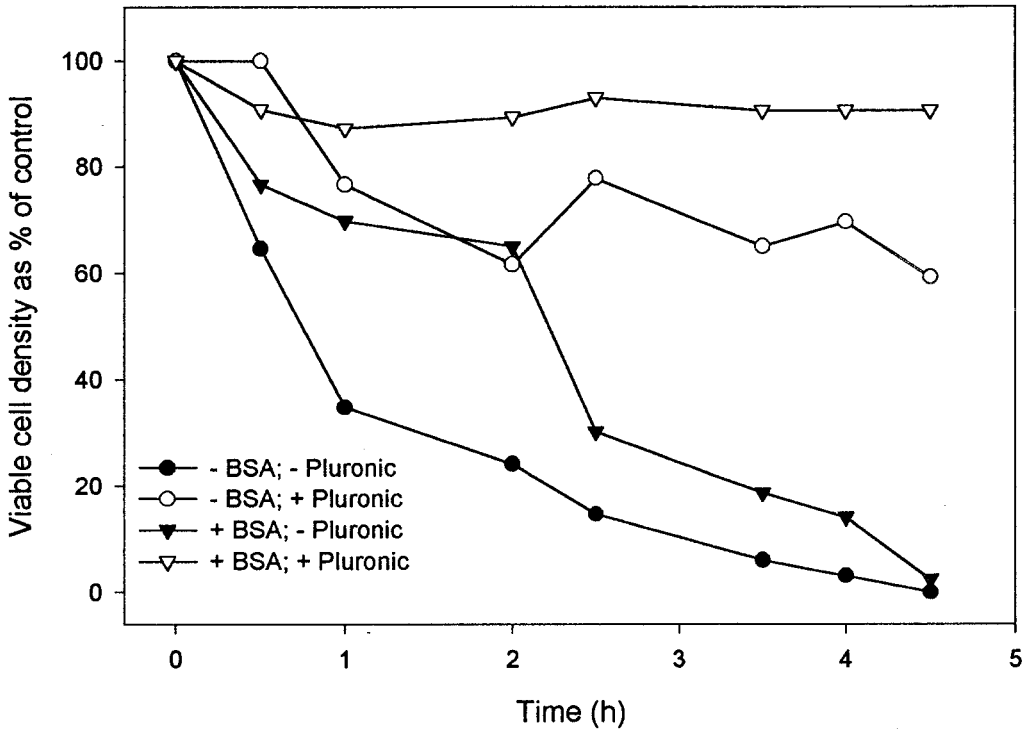

Figure 1. The effect of BSA and Pluronic on the the loss of cell viability at a high agitation rate. CC9C10 cells were inoculated at 5×10^5 cells/ml into 200 ml medium contained in a Bellco spinner flask which was agitated at 500 rpm for 4.5 h. The viable cell concentration was determined at intervals by haemocytometer counting in a trypan blue suspension. Cells grown with or without fatty acid-free bovine serum albumin (0.1 mg/ml) were supplemented with Pluronic (0.1%) 4 days prior to the experiment. Cultures contained the following supplements: none (●); Pluronic (○); BSA (▼); Pluronic and BSA (▽). Each point represents the mean of duplicate samples.

The effect of the rate of culture agitation on cell survival

The loss in cell viability was determined for cultures exposed to a range of agitation rates from 370 rpm to 630 rpm over a maximum period of 5 h. This agitation range corresponded to impeller tip speeds of 94 to 165 cm/sec. These rates are well above those used to grow cells and at all speeds within this range the cells are susceptible to the shear damage caused by the agitation of the culture. A distinct liquid vortex was observed in all these cultures.

Figure 3 shows the time-dependent decrease of the viable concentration of cells grown in cultures supplemented with fatty acid-free BSA. The cells had been grown for >5 passages in this medium prior to the experiment. The series of curves generated within this agitation range shows the increasing effect of impeller speed on the viability of the cells. The agitation rate of 370 rpm was the lowest at which a decline in cell viability was observed but only at rates above 470 rpm did the viable cell density decline below 50% of the original value within the time period of the experiment (5 h).

This experiment was repeated with cells that had been grown for 5 passages in media containing 25 μM linoleic acid (Figure 4). A similar series of curves was generated and again showing a decreased cell survival at higher agitation rates. However, the decline in viability of the linoleic acid-grown cells was less severe during agitation, particularly at stirring rates up to 550 rpm.

The data shown for agitation rates above 470 rpm in Figures 3 and 4 fit single exponential decay curves with the formula:

$$y = a\, e^{-bx} \qquad (1)$$

where a is the y value at x = 0, and b is a rate constant.

The constant values (a and b) were determined for each exponential decay curve by an algorithm contained in SigmaPlot 4.01. The half-life values from these curves were determined (= 0.69/b). These are plotted for the two cell populations in Figure 5. At the

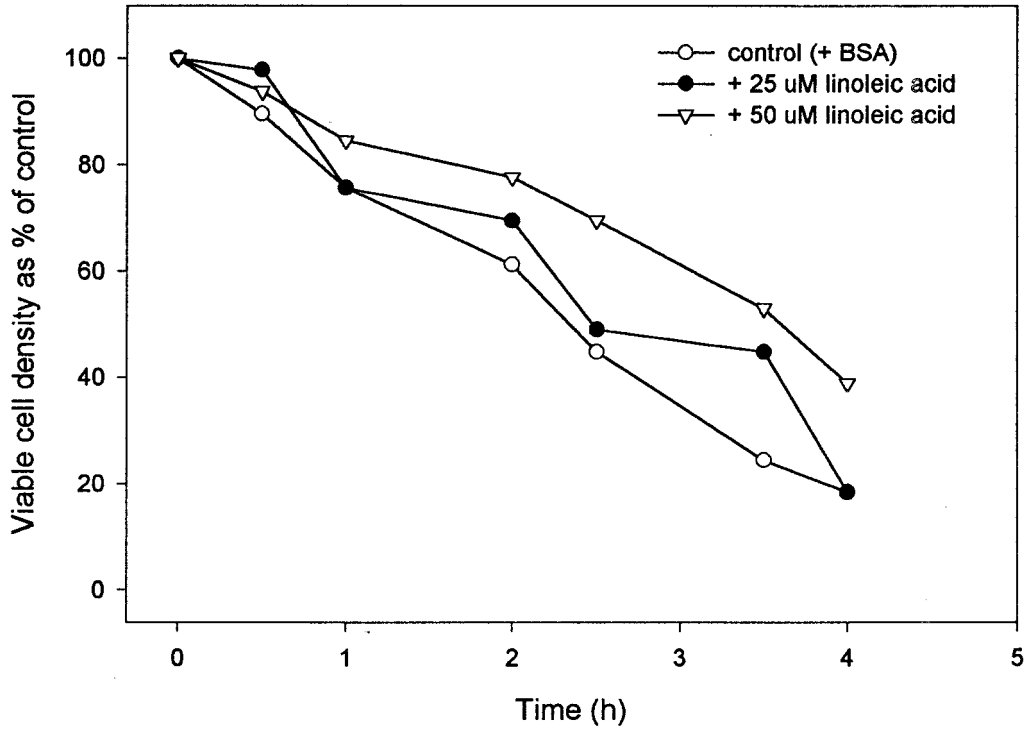

Figure 2. The effect of growth in linoleic acid over 1 culture passage. CC9C10 cells were grown for one passage in medium containing linoleic acid at 25 μM (●) or 50 μM (▽). The control cultures were supplemented with BSA (○). Each point represents the mean of duplicate samples.

highest agitation rate (630 rpm) the viable cell concentration decreased to zero after 1.5 h, whereas at the lowest rate (470 rpm) the viable cell concentration decreased to half the original value within 1 to 3 h. At 470 rpm the determined half-life for linoleic acid-grown cells was $\times 3$ the equivalent value of control cells. The half- life values determined for the linoleic acid-grown cells were significantly higher than those of control cells up to 550 rpm. At the higher agitation rates ($\geq$610 rpm) the half-lives were extremely short and no significant difference was found for the values for the two cell populations.

Discussion

Mammalian cells are commonly grown in stirred tank bioreactors because of their simplicity and ease of process control. The cultures are homogeneous and can be maintained at various levels of scale (1–1000 l) in such bioreactors. However, the relative fragility of animal cells compared to bacteria and fungi has necessitated specific design features such as round bottom fermenters and pitched blade or marine impellers to allow for sufficient mixing at low stirring rates (van der Pol et al., 1998). The survival of cells under such conditions is dependent not only on the stirring rate but also on the gas sparging or entrainment that might be necessary to supply oxygen (Handa-Corrigan et al., 1989).

The two types of fluid -mechanical shear stress that can cause cell damage are: (a) bubble disengagement caused by direct sparging or gas entrainment (b) liquid flow and turbulence caused by the impeller blade. In suspension cultures cell damage occurs typically at $>$200 rpm in stirred tank reactors and is associated with either bubble disengagement at the gas-liquid interface or bubble entrainment caused by vortex formation. In the absence of gas activity in the liquid, higher agitation rates ($>$700 rpm) are possible before cells are damaged by stresses in the bulk turbulent liquid. In microcarrier cultures damage to the anchorage-dependent mammalian cells by liquid turbulence occurs at lower agitation rates ($\sim$100 rpm). This has been correlated with the relative size of the turbulent eddies (Kolmogorov-scale) to the microcarrier size (Papoutsakis, 1991).

The shear sensitivity of cells in culture is dependent upon a number of factors including the growth

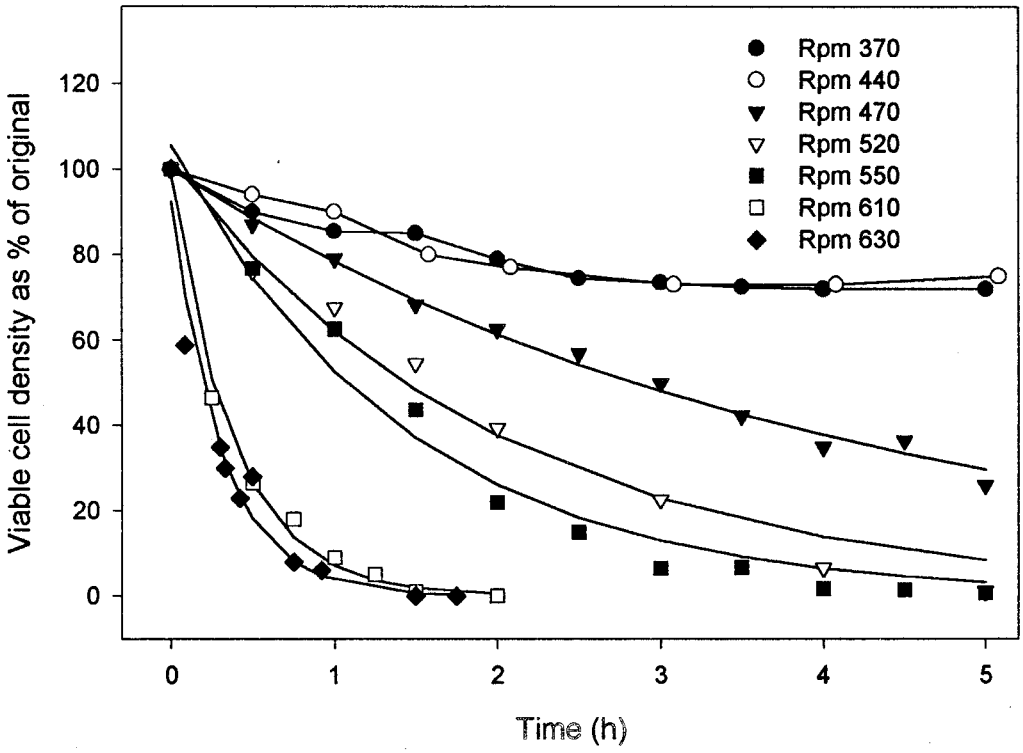

Figure 3. The effect of agitation on cultures grown without a fatty acid supplement. CC9C10 cells were grown in culture medium supplemented with BSA but in the absence of fatty acid. The cells were inoculated at 5×10^5 cells/ml into 200 ml medium contained in a spinner flask which was agitated at a selected speed from 370 to 630 rpm for 5 h. Agitation rates were 370 rpm (●); 440 rpm (○); 470 rpm (▼); 520 rpm (▽); 550 rpm (■); 610 rpm (■) and 630 rpm (♦). The viable cell concentration was determined at regular intervals over this period. Each point represents a mean value for three cultures.

phase, growth rate, culture age and media formulation (Petersen et al., 1988; Martens et al., 1993; Al-Rubeai et al., 1995). A number of additives have been shown to increase the survival of cells in agitated cultures. These additives include serum, albumin and the non-ionic surfactant, Pluronic F-68 (Michaels et al., 1991; Al-Rubeai et al., 1995). Two protective mechanisms have been proposed. Firstly, a biological mechanism causes changes in the ability of cells to resist shear. This occurs generally by metabolic changes occuring over a relatively long period. Secondly, a physical mechanism reduces the level and frequency of shear forces experienced by the cells.

Cell protecting additives, such as Pluronic F-68 are commonly included as a media component in order to reduce cell damage in gas sparged, agitated cultures. Shear protection occurs mainly by a physical mechanism brought about by a reduction in the cell to gas bubble attachment (Kunas and Papoutsakis, 1990; Jordan et al., 1994). The protective effect of serum is largely physical but may have a minor biological component which may be associated with the presence of key micro-nutrients (Michaels et al., 1995).

In our agitation assays in spinner flasks reported here for the CC9C10 cells, we use stirring rates that cause a vortex in the liquid. This causes gas entrainment and the type of cell damage characteristic of sparged stirred tank bioreactors. The protective effect of both albumin and Pluronic was shown to be substantial. In the presence of the two additives cell damage was minimal at 500 rpm, whereas in their absence the viability of the cells was reduced to 50% in 50 min.

The multiple metabolic effects of a supplement of an unsaturated fatty acid on cultures of CC9C10 cells has been described previously (Butler et al., 1997). Micromolar concentrations of the unsaturated fatty acids, oleic and linoleic causes substantial effects, which include a significant growth enhancement (300%), an initial enhancement of Mab yield (60%) and a significant reduction in glutamine metabolism (60%) (Butler et al., 1997).

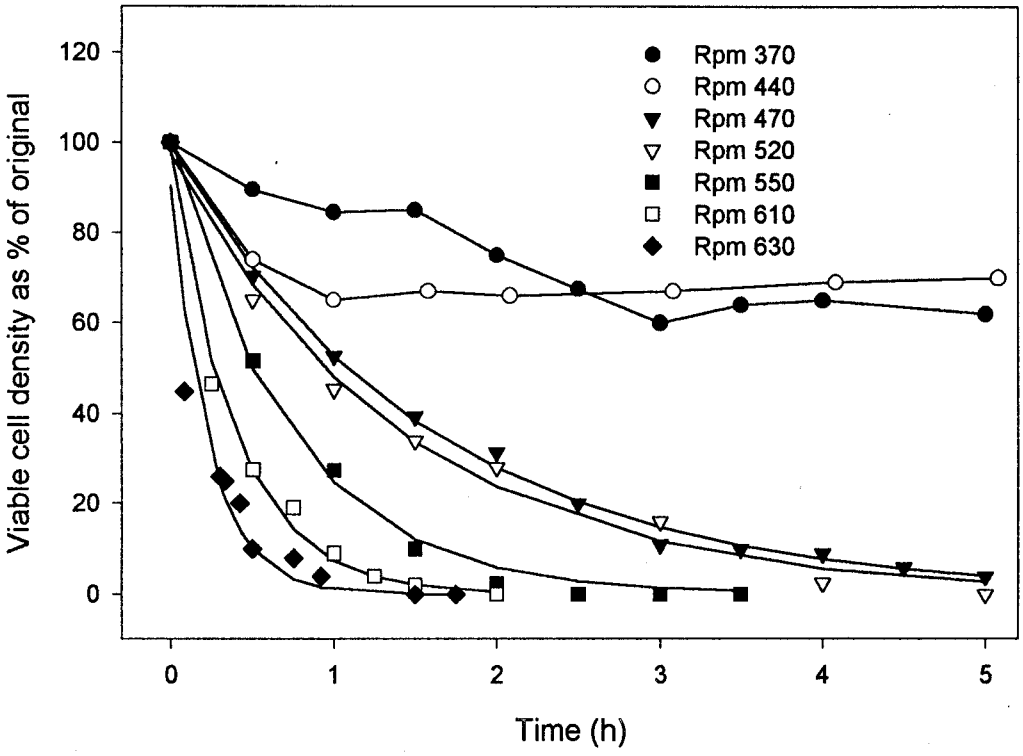

Figure 4. The effect of agitation on cultures grown in the presence of linoleic acid. CC9C10 cells were grown with 25 μM linoleic acid for at least 5 passages prior to the start of the experiment. The experimental conditions and measurements were as indicated in Figure 3.

There may be several mechanisms for these metabolic effects. Unsaturated fatty acids have been shown to directly activate specific enzymes such as phospholipases (Gillham and Brindle, 1996) which may in turn activate protein kinase in a signal transduction mechanism. Such a mechanism may be associated with the induction of DNA synthesis and cytokine release from lymphocytes under physiological regulation (Karsten et al., 1994). The enhancement of specific Mab productivity suggests that linoleic acid may also have a direct effect on the synthetic pathways of hybridomas in culture. This may also explain the observed change in the kinetics of glutamine uptake. The altered characteristics of glutamine uptake seemed to be the primary factor in causing a perturbation in the pattern of energy metabolism. The $\times 10$ increase in Km for glutamine uptake in the presence of linoleic acid suggests the possibility of the synthesis of an alternative glutamine transporter (Butler et al., 1997). This may be produced by an induction of specific protein synthesis.

Of particular relevance to the robustness of the cells is the relatively high incorporation of the available fatty acids into the cellular phospholipid fraction which is a major structural component of the outer membrane of the cell. This may explain the apparent increase in the robustness of the fatty acid-grown hybridomas in agitated cultures. An altered unsaturated/saturated fatty acid ratio has been shown previously to lead to changes in some physical properties such as membrane fluidity (Calder et al., 1994). There is also a relationship between membrane fluidity and shear sensitivity of cells (Ramirez and Mutharasan, 1990). The possibility that these changes could lead to an improved cellular robustness was the rationale for the experiments described in this paper.

In the simple assay developed to monitor cell survival, a measurable rate of loss of cell viability was observed only at 370 rpm. A stirring rate of 370–440 rpm reduced the cell viability of linoleic acid-grown cells by 20% as compared to control values which were reduced by 30–40% over the same period of 5 h. The clearest difference in the survivability was observed between 470–550 rpm when the half-life of the linoleic acid-grow cells was up to $\times 3$ greater than the control values. At 610 rpm the loss of cell

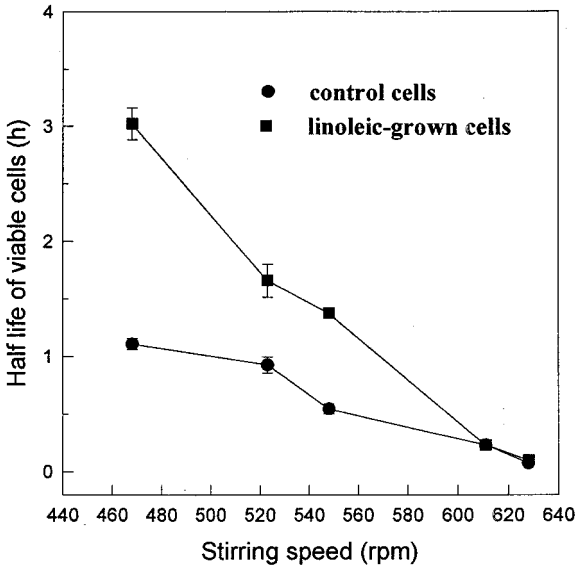

Figure 5. The effect of stirrer speed on the half-life of cells in agitated culture. CC9C10 cells were inoculated at 5×10^5 cells/ml into 200 ml medium contained in a spinner flask which was agitated at selected speeds for 5 h grown in the absence of fatty acids (●) and for cells grown with 25 μM linoleic acid (■) for at least 5 passages. The half-life of the viable cell population at various agitation speeds is shown over the range 470–630 rpm. Values are means (±SEM) for three cultures.

viability was extremely rapid and no significant difference was observed between the cell populations. This increased survivability of cells occured following 5 passages of growth in 25 μM linoleic acid. At 1 passage, an increased concentration (50 μM) of linoleic acid was necessary before a significant change was observed. This is compatible with the explanation of an increased cellular robustness brought about by incorporation of linoleic acid into the cellular membrane. It was shown previously that the level of cellular incorporation of supplemented unsaturated fatty acids increased over several passages (Butler et al., 1997).

The regulatory mechanism of cellular uptake of fatty acids appears to be limited and so the composition of the intracellular lipids is likely to reflect the availability of the fatty acids in the medium. This was shown for the CC9C10 hybridomas (Butler et al., 1997) and by others for BHK and CHO cells (Schmid et al. (1991). Thus, cells grown in serum-supplemented cultures are likely to attain a fatty acid composition reflecting that of serum, in which the predominant fatty acids of serum are palmitic, stearic, oleic and linoleic acids at a ratio of 2:1:3:1, respectively. This ratio has been implicated as being important for the physiological regulation of human lymphocytes (Karsten et al., 1994). These were also the four predominant fatty acids measured in CC9C10 cells but with an initial ratio of 0.9:1:1:0.2 (p/s/o/l), indicating a considerably higher saturated fatty acid content (Butler et al., 1997). An optimal intracellular saturated/unsaturated fatty acid ratio for cell growth was proposed by Doi et al (1978) who showed that normal growth of LM cells could be maintained if the unsaturated content of the membrane phospholipid fraction was >56%. This is compatible with the observed increase in growth of the CC9C10 cells following initial addition of linoleic acid to the cultures, which increased the unsaturated content of the cellular lipids from 32 to 88% after 1 passage (Butler et al., 1997).

The availability of the unsaturated fatty acids in the culture medium appears to be a major factor controlling the lipid content of the cells and accounts for the three metabolic states – lipid-starved, lipid-balanced and lipid-loaded. For maximum productivity the fatty acid composition of the cell requires to be finely balanced between a lipid-starved and lipid-loaded state. As there appears to be no or only a limited regulatory mechanism for cellular uptake of fatty acids, the lipid-balanced state of the cells has to be controlled by a carefully designed feeding regime. For a production process, continuously transferring cells from lipid to lipid-free media would clearly be undesirable. Therefore, it is important to establish a pattern of systematic supplementation of unsaturated fatty acids during cell growth. Alternatively, sequential serum-free cultures could be designed with a fatty acid supplement that was sufficiently low to prevent adverse effects from cellular accumulation but sufficiently high to stimulate productivity over the course of the planned cultures.

The significance of the work described here is to show that it is possible to increase the resistance of cells to shear forces by alterations in the micronutrient content of the medium. Such a strategy may offer an alternative to the addition of shear protectants to the cultures. Despite the value of Pluronic in protecting cells, there is a potential problem in that Pluronic may have some cytotoxic effects which have been documented for certain cell lines (Handa-Corrigan et al., 1997). The cytotoxic effects of Pluronic may be undesirable for two reasons. Firstly, it may reduce the yield of the producer cell line in culture and secondly there is a possibility of complexation or co-purification with a cell product. The latter could lead to undesirable side-effects in administration of a therapeutic products. These factors suggest

an incentive for the development of alternative methods to protect producer cell lines in agitated cultures. The advantage of more robust producer cell lines is that they will allow higher stirring or sparging rates in stirred tank bioreactors. This in turn improves the possibilities for mixing and oxygen supply in large-scale cultures.

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